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Toxicology and Applied Pharmacology 191 (2003) 95–106

Toxicology and Applied Pharmacology

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Ontogenic differences in human liver 4-hydroxynonenal detoxification are associated with in vitro injury to fetal hematopoietic stem cells

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Received 19 November 2002; accepted 29 April 2003

Abstract

4-hydroxynonenal (4HNE) is a highly mutagenic and cytotoxic α,β-unsaturated aldehyde that can be produced in utero during transplacental exposure to prooxidant compounds. Cellular protection against 4HNE injury is provided by alcohol dehydrogenases (ADH), aldehyde reductases (ALRD), aldehyde dehydrogenases (ALDH), and glutathione *S*-transferases (GST). In the present study, we examined the comparative detoxification of 4HNE by aldehyde-metabolizing enzymes in a panel of adult and second-trimester prenatal liver tissues and report the toxicological ramifications of ontogenic 4HNE detoxification in vitro. The initial rates of 4HNE oxidation and reduction were two- to fivefold lower in prenatal liver subcellular fractions as compared to adult liver, and the rates of GST conjugation of 4HNE were not detectable in either prenatal or adult cytosolic fractions. GSH-affinity purification of hepatic cytosol yielded detectable and roughly equivalent rates of GST–4HNE conjugation for the two age groups. Consistent with the inefficient oxidative and reductive metabolism of 4HNE in prenatal liver, cytosolic fractions prepared from prenatal liver exhibited a decreased ability to protect against 4HNE-protein adduct formation relative to adults. Prenatal liver hematopoietic stem cells (HSC), which constitute a significant percentage of prenatal liver cell populations, exhibited ALDH activities toward 4HNE, but little reductive or conjugative capacity toward 4HNE through ALRD, ADH, and GST. Cultured HSC exposed to 5 μM 4HNE exhibited a loss in viability and readily formed one or more high molecular weight 4HNE-protein adduct(s). Collectively, our results indicate that second trimester prenatal liver has a lower ability to detoxify 4HNE relative to adults, and that the inefficient detoxification of 4HNE underlies an increased susceptibility to 4HNE injury in sensitive prenatal hepatic cell targets.

Keywords: 4-Hydroxynon-2-enal metabolism; Human liver; Ontogeny; Hematopoietic stem cells

Introduction

Under conditions of oxidative stress, a number of breakdown products are released that decompose to produce locally high concentrations of highly reactive α,β-unsaturated aldehydes. In particular, 4-hydroxynon-2-enal (4HNE) is a potent mutagen released during lipid peroxidation which readily forms covalent adducts with DNA and proteins (Esterbauer et al., 1991). Conditions that elevate cellular 4HNE result in loss of mitochondrial function and cell necrosis, often accompanied by a loss of viability through apoptosis (Ramachandran et al., 2001). Coincidently, elevated tissue 4HNE concentrations have been associated with several human diseases, including cancer (Eckl et al., 1993), Parkinson's disease, Alzheimer's disease (Markesbery and Lovell, 1998), atherosclerosis (Chen et al., 1995; Muller et al., 1996), pulmonary inflammation (Hamilton et al., 1996), rheumatoid arthritis (Selley et al., 1992), and ophthalmologic disorders (Esterbauer et al., 1991). There is also strong evidence to indicate that the in utero production of aldehydes are important in the oxidative pathogenesis of a variety of perinatal diseases. For example, a linkage has been made between the appearance of 4HNE and perinatal

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doi:10.1016/S0041-008X(03)00220-5

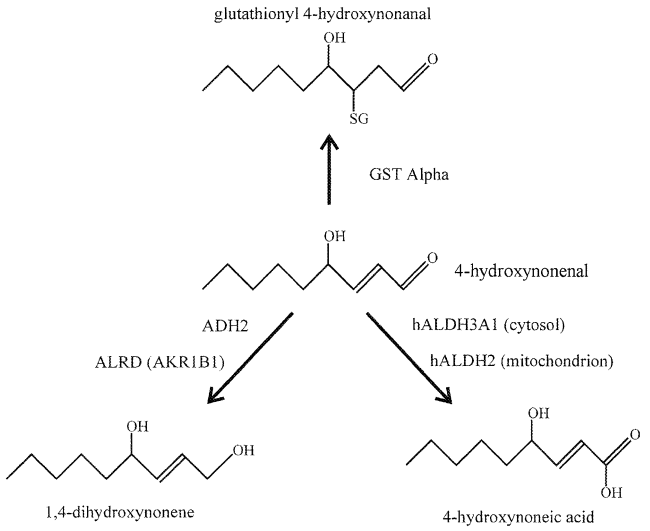

Fig. 1. Primary enzymatic pathways of 4HNE metabolism in human liver.

chronic lung disease (Ogihara et al., 1999) as well as perinatal hypoxia (Schmidt et al., 1996). There is also a causal relationship between the in utero production of 4HNE or MDA and the prenatal hepatotoxicity of in utero ethanol exposure (Chen et al., 1997b; Hartley and Petersen, 1997; Lieber, 1997; Henderson et al., 1999; Chen et al., 2000; Heaton et al., 2000; Ramachandran et al., 2001). Other studies have shown that critical developmental biochemical pathways may be affected by alterations in the intracellular levels of 4HNE (Soprano and Soprano, 1995; Khalighi et al., 1999). Collectively, the results of these and other studies demonstrate that 4HNE plays an important role in the etiology of many developmental and adult diseases.

Given the high reactivity and toxicological importance of 4HNE, it is not surprising that a number of enzyme systems have evolved to protect tissues from 4HNE injury (Esterbauer et al., 1991). The primary enzymatic pathways of 4HNE detoxification in adult human liver include aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), aldehyde reductase (ALRD), and glutathione *S*-transferase (GST; Fig. 1). Kinetic studies indicate that ALDH1 is likely the most important cytosolic ALDH isoform responsible for 4HNE oxidation in rat liver (Mitchell and Petersen, 1987; Marselos and Lindahl, 1988). In humans, however, class 3 ALDH (*ALDH3A1* and *ALDH3A2* appear to be the primary pathway of 4HNE oxidation to 4-HNE in liver cytosol (Lindahl and Petersen, 1991; Vasiliou et al., 2000; Townsend et al., 2001), exhibiting a K_m = 110 μM and k_{cat}/K_m = 0.40 $s^{-1}\mu M^{-1}$ (King and Holmes, 1993). Although the class I ALDH1A1 also metabolizes 4HNE, overexpression of ALDH1A1 affords little protection against medium chain-length aldehyde substrates, including 4HNE (Townsend et al., 2001). The *ALDH2* gene encodes a mitochondrial enzyme that is highly expressed in human liver and is also an important contributor to 4HNE oxidation (Vasiliou et al., 2000). Human liver ADH and ALRD enzyme families are active in the reduction of 4HNE to 1,4-dihydroxy-2-nonene (Sellin et al., 1991). In this regard, ADH2 constitutes a particularly important ADH isozyme in 4HNE metabolism and exhibits a K_m = 97 μM and k_{cat}/K_m = 0.46 $s^{-1}\mu M^{-1}$ (Duester et al., 1999). The ALRD subtype AKR1B1 exhibits a K_m = 22 μM and k_{cat}/K_m = 0.077 $s^{-1}\mu M^{-1}$ for 4HNE reduction (O'Connor et al., 1999). The alpha class GSTs, of which hGSTA1-1 is the most abundant form in human liver cytosol, are actively involved in 4HNE detoxification through conjugation with glutathione (GSH). In particular, hGSTA1-1 conjugates 4HNE with a K_m = 50 μM and a k_{cat}/K_m = 0.058 $s^{-1}\mu M^{-1}$, whereas hGSTA4-4, a primarily mitochondrial GST (Gardner and Gallagher, 2001) effectively conjugates 4HNE with an efficient K_m = 49 μM and a very rapid k_{cat}/K_m of 2.7 $s^{-1}\mu M^{-1}$ (Cheng et al., 2001).

Despite the demonstrated importance of these enzymes in protecting against 4HNE injury in human adult and in rodent tissues, little is known regarding the expression and efficiency of 4HNE-metabolizing enzymes in human prenatal tissues. The ontogenic expression of prenatal detoxification pathways is particularly important as a number of transplacental toxicants, including cyclophosphamide, acetaminophen, phenytoin, and benzo[*a*]pyrene, are capable of promoting oxidative injury in utero that may result in disease outcomes that are manifested in childhood (Wells and Winn, 1996; Sasco and Vainio, 1999; Bialkowska et al., 2000). Of particular concern are critical developmental cell populations such as potential hematopoietic stem cells, which may be targets for maternally transferred chemicals. In this regard, epidemiological studies have demonstrated an association among the occurrence of nonfunctional alleles for certain polymorphic detoxification genes (e.g., *hGSTM1* and *hGSTT1*) and the increased risk for developing acute lymphoblastic leukemia (ALL) (Chen et al., 1997a; Krajinovic et al., 1999). The long latency period for cancer development and the toxicological basis for certain childhood diseases underscores the importance of understanding, in utero gene–environment interactions in the context of 4HNE detoxification.

In the current study, we tested the hypothesis that human prenatal liver and its associated hematopoietic stem cells (HSC) are highly sensitive to in vitro 4HNE injury. We report the comparative rates of 4HNE biotransformation in prenatal and adult human liver tissues and HSC through ADH, ALRD, ALDH, and GST, and the toxicological ramifications of ontogenic enzyme expression. Of particular interest was that the comparative inefficiency of 4HNE detoxification through these enzymatic pathways was associated with a lesser ability to attenuate 4HNE–protein adduct formation in human prenatal liver. Moreover, we demonstrate that cultured hu-

man prenatal liver HSC are highly sensitive to 4HNE injury and 4HNE–protein adduct formation.

Materials and methods

Chemicals. 4HNE was purchased from Cayman Chemical (Ann Arbor, MI). Iscove's modified Dulbecco's medium (IMDM), penicillin, streptomycin, and heat-inactivated fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Recombinant human interleukin 3 (IL-3), granulocyte colony stimulating factor (G-CSF), and stem cell factor (SCF) were obtained from R&D Systems (Minneapolis, MN). Cryopreserved Poietics $CD34^+$ human prenatal liver cells of 97.4% purity were purchased from BioWhittaker (Walkersville, MD). Alamar Blue was purchased from BioSource International (Camarillo, CA). Anti reduced 4HNE protein antibodies were purchased from Calbiochem (San Diego, CA). Western blotting luminol reagent and horseradish peroxidase-linked goat antirabbit IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Orlando, FL).

Tissue samples and subcellular fractionation. All human tissue work was approved by the University of Florida Health Center Institutional Review Board. Second trimester prenatal liver samples consisted primarily of Caucasian donors, ages 20–24 weeks and were obtained from the Anatomical Gift Foundation (AGF, White Oak, GA), an independent nonprofit human tissue bank that provides tissue for biomedical research. All prenatal livers were obtained through elective termination of pregnancy. Because the specimens obtained by the tissue banks would otherwise be discarded and are from anonymous donors, these studies are exempted by regulatory provisions of human subject research (45 CFR 46). Adult human liver samples were also obtained from AGF and were derived from nonsmoking Caucasian male donors without a history of drug abuse. Strict confidentiality of donor information was maintained for all human samples. Mitochondrial and cytosolic fractions were isolated as previously described (Gardner and Gallagher, 2001). LDH activity in mitochondrial fractions was used as a measure of potential cytosolic contamination of the mitochondrial preparations (Statham et al., 1977). All subcellular fractions were stored at −80°C until use. Protein concentrations were determined using the bicinchoninic acid (BCA) method (Smith et al., 1985) with bovine serum albumin as a standard.

Affinity purification of adult and prenatal liver cytosolic GST. Five-micromolar concentrations of dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride were added to 600 μl of liver cytosol prior to incubation with 60 μl GSH linked to Sepharose 4B matrix (Pharmacia, Uppsala, Sweden). The cytosol and GSH matrix were mixed at room temperature for 10 min in a spin column and washed twice with 0.5 ml Tris-buffered saline (TBS). Bound protein was eluted with 150 μl of 50 mM Tris–HCl, 150 mM glutathione, and 1.4 mM β-mercaptoethanol, pH 9.6. Eluate alkalinity was immediately adjusted to neutrality with the addition of 100 mM Tris–HCl, pH 7.2. Samples were then dialyzed for 40 h at 4°C against 500 volumes of TBS, with two changes of dialysis buffer.

Initial rate of 4HNE metabolism by ADH, ALDH, ALRD, and GST in human liver subcellular fractions and hematopoietic stem cells. The comparative metabolism of 4HNE in adult fetal liver subcellular fractions was conducted using similar 4HNE substrate concentrations (100 μM) so that the relative contributions of the pathways could be compared. Although a substrate concentration of 100 μM 4HNE is somewhat above physiological 4HNE concentrations, it is nonsaturating and allows for comparative detection of the major 4HNE metabolites in human prenatal and adult samples. All assays were conducted in triplicate with the appropriate nonenzymatic rates of 4HNE metabolism subtracted from the enzymatic reactions.. The initial rates for ALDH oxidation of 4HNE were determined essentially as previously described (Canuto et al., 1994) with minor modifications. Briefly, the reaction mixture consisted of 100 mM Tris (pH 7.4), 1 mM NAD+, 100 μM 4HNE, 0.5–1 μg protein, 2 mM pyrazole, and 2 μM rotenone. Protein samples were incubated with pyrazole, rotenone, and, in the case of mitochondrial samples, with 0.1% Triton X-100 on ice for 10 min. The reaction mixture, without 4HNE, was added to a 96-well plate, protein was added, and the rate of NAD^+ reduction was monitored at 340 nm for 5 min in order to ensure the complete inhibition of alcohol dehydrogenase and NADH oxidase. After addition of 4HNE, the rate of NAD^+ reduction was monitored for 20 min at 25°C. Because 4HNE can react with free of means and Tris buffers and form Schiff bases (personal communication with Dr. Dennis Petersen, University of Colorado), a subset of samples were used to compare the rates of ALDH-4HNE metabolism in the presence of sodium phosphate and Tris buffers. The reduction of 4HNE by ADH and ALRD was measured as previously described (Canuto et al., 1994). GST activity toward 4HNE was determined by the spectrophotometric method of Singhal et al. (1994) with minormodifications. Due to high background absorbance by interfering proteins at 224 nm, GSH affinity-purified liver cytosolic fractions as well as cytosolic fractions were used as the enzymatic source. The reaction mixtures (final volume 1 ml) contained 100 mM KPO_4 (pH 6.5), 0.5 mM GSH, 25 μl of purified GST or cytosol, and 0.1 mM 4HNE. The reactions were initiated by the addition of 4HNE, and its utilization was monitored at 224 nm in a Cary dual beam

spectrophotometer against a blank that contained all of the reactants except 4HNE. Assays were conducted at 30°C and were corrected for nonenzymatic activity and nonspecific binding of 4 HNE using heat-inactivated subcellular fractions.

In vitro inhibition of 4HNE-BSA adduct formation by adult and prenatal cytosol. In order to assess the relative degree of protection from 4HNE-protein alkylation afforded by human adult and prenatal liver, BSA was incubated with 4HNE in the absence or presence of varying amounts of adult and prenatal cytosol. Attenuation of 4HNE-protein adduction was monitored immunologically. Using a 96-well plate, wells were filled with 80 μl of 37.5 mM KPO_4, 1.25 mg/ml (100 μg) bovine serum albumin (BSA), with 0.125 mM each of the enzymatic cofactors GSH, NADPH, NADH, and NAD^+ at pH 7.4. Ten microliters (1–20 μg) of cytosolic protein pooled from four adult or four prenatal samples were added in quadruplicate to the wells. The adult and prenatal subcellular fractions were selected based on reflection of representing typical differences in 4HNE detoxification rates among the panel for the two respective age groups. Negative controls (lacking cytosol or lacking cofactor) were also included on each plate. In some experiments, the contributions of GST toward 4HNE protection was assessed by carrying out reactions in the absence of GSH, and using heat-inactivated cytosolic fractions in the presence or absence of GSH. The plate was incubated at 37°C for 5 min, and the reactions were initiated by the addition of 10 μl of 1 mM 4HNE (final assay concentration of 100 μM 4HNE). After shaking, the reactions were allowed to proceed for 20 min at 37°C. During this time the Gibco BRL Hybridot blotting apparatus was assembled containing Whatman filter paper and PVDF membrane equilibrated with methanol followed by TBS. Wells of the blotter were flushed with 300 μl TBS prior to loading protein. Immediately after the 20-min incubation period, 12 μl from each well was diluted to a final volume of 300 μl (0.04 mg/ml BSA) and 125 μl (5 μg BSA) added to the wells of a blotting apparatus. Vacuum was applied, and wells were flushed with an additional 300 μl TBS, the blotter was disassembled and vacuum turned off. 4HNE-protein adducts were detected through the use of an antibody which specifically recognizes the reduced 4HNE–protein adduct (Neely et al., 1999). Immediately after immobilizing the proteins, 4HNE–protein adducts were reduced by soaking the membrane in TBS containing 10 mM sodium borohydride for 30 min at 23°C. Subsequent blocking and antibody incubations were performed as described previously (Montine et al., 1998; Neely et al., 1999) with the exception that the horseradish peroxidase-linked goat antirabbit IgG secondary antibody was used at a dilution of 1:2000. Imaging and quantitation was performed using the Bio-Rad Fluor-S Multi-Imager with Quantity One software. Measurement of total 4HNE–protein adduct formation was accomplished by quantitating the sum of pixel intensities (counts or cnt) inside the volume boundary area of a given coordinate (in square millimeters). For background subtraction, background intensity volume consisted of an annulus of fixed area surrounding each measured coordinate. In addition, the pixel intensities of the values obtained for nonenzymatic protection differences (e.g., cytosolic proteins in the absence of cofactors) were subtracted from the enzymatic values.

4HNE–protein adduct formation and toxicity in cultured HSC. In order to discern the sensitivity of HSC to 4HNE, cell viability was monitored by Alamar blue reduction in cultured $CD34^+$ stem cells treated with physiologically relevant levels of 4HNE. $CD34^+$ cells from human prenatal liver were seeded at 25,000 cells/ml of IMDM containing 15% FBS, 2 ng/ml IL-3, 1 ng/ml G-CSF, 20 ng/ml SCF, and 100 U penicillin/ml, 100 μg streptomycin/ml, and cultured for 10 days following conditions of Warren et al. (1995). Under, these culture conditions, $CD34^+$ cells derived from human prenatal liver undergo moderate proliferation and differentiate as a mixed population of HSC ($CD34^+$), monocytes ($CD14^+$), neutrophils ($CD15^+$), and megakaryocytes ($CD41^+$) (Warren et al., 1995). Cell viability was measured using trypan blue exclusion, and 20 ml of fresh media were added to the culture. At the end of the culture period, cells were transferred to 6-well Costar plates (Corning Inc., Corning, NY), and seeded at 1,200,000 cells per well, in a total volume of 3 ml. Treatments consisted of untreated control, vehicle control (1% ethanol), 5 μM 4-HNE, and 50 μM 4-HNE. Immediately following treatment, 10% Alamar blue was added to each well. The effects of 4 HNE on proliferation of HSC was quantitated at 1 h and 2 h postexposure by fluorescence measurement of Alamar blue reduction at an excitation wavelength of 544 nm and an emission wavelength 590 nm (Ahmed et al., 1994). Cultured $CD34^+$ cells treated with 4HNE for 2 h were collected after the Alamar blue assay by centrifugation at 600*g* for 10 min. Cellular pellets were lysed using 0.5% SDS, and protein content was determined by the BCA method. Twenty micrograms of protein from each treatment group were loaded onto a Bio-Rad Criterion 8 to 16% gradient polyacrylamide gel and size fractionated as instructed by the manufacturer. Proteins were then transferred to PVDF membrane using a Bio-Rad semidry electroblotter as instructed by the manufacturer and 4HNE–protein adducts were detected as described above. Aliquots of untreated cells were snap frozen in liquid nitrogen and transferred to −80°C for subsequent preparation of subcellular fractions and enzymatic activities as described above.

Statistical analysis. Initial rate enzyme activities represent the average of a minimum of triplicate incubations. Sig-

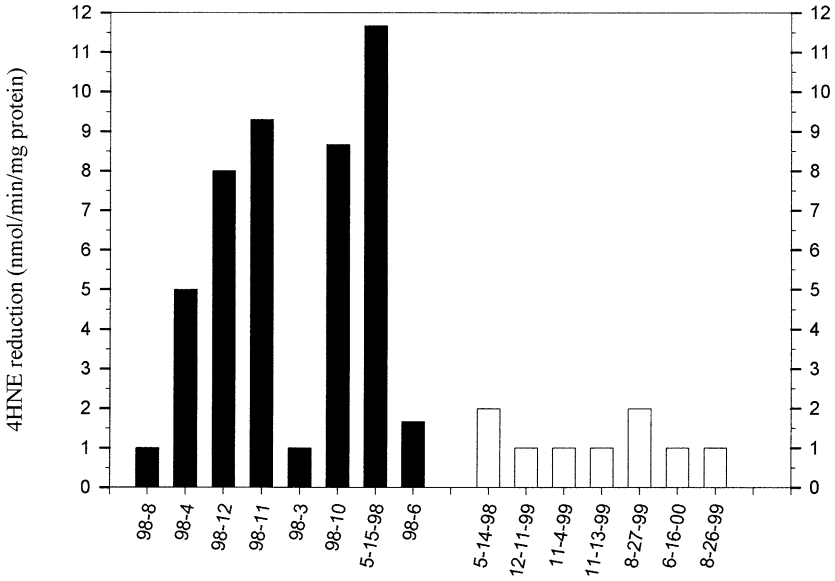

Fig. 2. Comparison of initial rates of cytosolic ADH-4HNE reduction in a panel of human prenatal liver donors (white bars) with human adult donors (black bars). All assays were carried out in triplicate and corrected for nonenzymatic activity as described under Materials and Methods. The mean ADH-4HNE activities were significantly lower in prenatal donors as compared to adults ($P \leq 0.05$).

nificant differences in enzymatic activities among the adult and prenatal age groups were determined using an unpaired Student's *t* test and a probability value of $P \leq 0.05$ (Statview 4.5, Abacus Concepts, Berkeley, CA).

Results

Comparative detoxification of 4HNE in prenatal and adult liver subcellular fractions

The mean initial rate of cytosolic ADH-mediated 4HNE reduction in adult liver (5.9 ± 4.0 nmol/min/mg) was 4.5-fold higher than that observed in second trimester prenatal liver (1.3 ± 0.5 nmol/min/mg) (Fig. 2). The mean initial rates of cytosolic 4HNE reduction by ALRD were relatively slow in both age groups (1.6 ± 0.2 nmol/min/mg in adults and 0.9 ± 0.2 nmol/min/mg in prenatal donors; Fig. 3). Despite the relatively slow initial rates of ALRD-mediated 4HNE reduction in all samples, ALRD-4HNE activities were significantly higher in adult liver as compared to prenatal liver. ALRD-mediated 4HNE reductase activities showed extensive interindividual variation and among both age groups (range of 0.7–4.2 nmol/min/mg in adults and 0.3–1.8 nmol/min/mg in prenatal liver). Similarly, a substantial (12-fold) interindividual difference in ADH-4HNE activities were observed among adults.

Figure 4A reveals significantly higher cytosolic ALDH-4HNE oxidase activities in adult liver tissue (mean of 5.9 ± 0.41 nmol/min/mg) as compared to prenatal liver tissues (0.89 ± 0.14 nmol/min/mg). A similar pattern was observed in human liver mitochondrial ALDH (Fig. 4B). A minor (<30%) reduction in ALDH-4 HNE activities was observed in the presence of Tris buffer as compared to sodium phosphate. However these results did not affect the magnitude of differences observed among age groups or interpretation of the data. In initial studies, we were unable to reliably detect GST-4HNE conjugation in either human or adult liver cytosol, possibly due to a relatively high assay limit of detection (10 nmol/min/mg protein; Fig. 5A) due to protein intereferences at 224 nm. However, as observed in Fig. 5B, enrichment of GST by affinity purification yielded mean rates for adult and prenatal affinity-purified GST-4HNE conjugative activities were 2900 ± 340 and 2200 ± 270 nmol/min/mg protein respectively and did not significantly differ among the two age groups. Extensive variation was observed among adult GST-4HNE conjugative activities (9-fold variation) and among prenatal GST-4HNE activities (5-fold variation).

Comparative detoxification of 4HNE by prenatal liver HSC

The initial rates of ADH, ALRD, ALDH and GST-4HNE metabolism in HSC are presented in Table 1. As observed, ALDH-mediated 4HNE oxidation was observed in both S-9 and mitochondrial fractions prepared from HSC. In contrast, HSC did not readily detoxify 4HNE through ALRD or ADH reductive pathways (practical detection limit of reductive assays 0.1 nmol/min/mg). Furthermore, cystolic GST-4HNE conjugation activity was not present at detectable

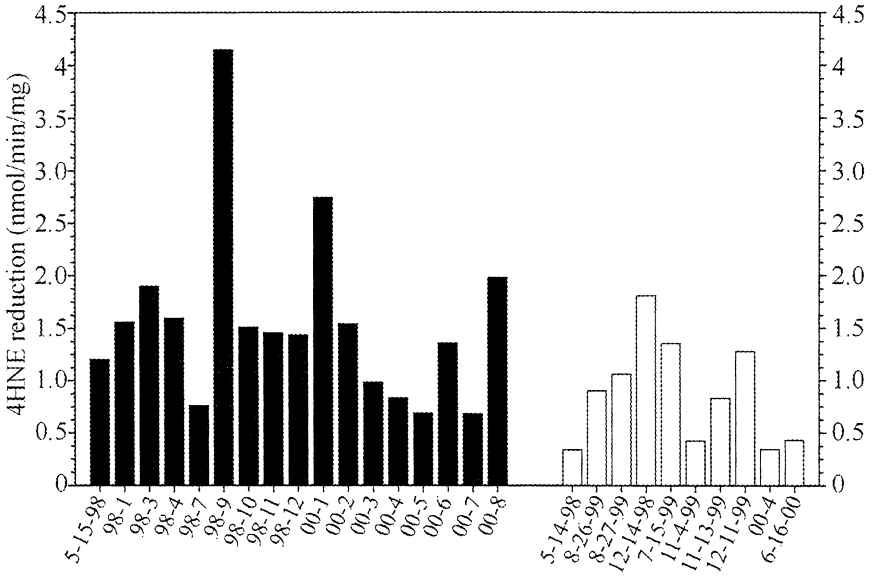

Fig. 3. Comparison of initial rates of cytosolic ALRD-4HNE reduction in a panel of human prenatal liver donors (white bars) with human adult adult donors (black bars). All assays were carried out in triplicate and corrected for nonenzymatic activity as described under Materials and Methods. The mean initial rates of ALRD-4HNE activities did not significantly differ in prenatal donors as compared to adults ($P \leq 0.05$).

levels (practical detection limit of GST-4HNE assay of 10 nmol/min/mg).

Attenuation of 4HNE–protein adduct formation by prenatal and adult cytosolic protein

The biological consequence of the lower capacity for 4HNE detoxification in prenatal tissues was tested by comparing the ability of adult and prenatal liver fractions to attenuate 4HNE–protein adduct formation in vitro. Conditions for detection of 4HNE adducts in a dot blot were initially established with adult cytosolic fractions. The time course during which 4HNE and cofactor concentrations were used for experiments here was chosen to minimize the nonenzymatic conjugation of GSH to 4HNE as determined by the degree of attenuation of 4HNE–BSA conjugation via cofactor alone. BSA concentrations were chosen in order to maintain a high concentration (5- to 100-fold) of inert, target protein relative to cytosolic protein. Cytosolic protein concentrations were chosen based on ability to discern linear changes in immunochemical intensity. Figure 6 illustrates the outcome of these experiments. Over a concentration range of 1–20 μg cytosolic protein, fetal liver was consistently less efficient in attenuation of 4HNE-protein adduct formation, relative to adult liver. When 20 μg of cytosolic protein added, 4HNE-protein adduct formation was 8.5-fold higher in the presence of fetal liver cytosol as compared to adult liver cytosol (Fig. 6A). Omission of GSH, or heat inactivation of cytosolic fractions in the presence of GSH, abolished the 4HNE protective capacity of fetal liver cytosol, and substantially reduced the protective capacity of adult liver cytosol (Fig. 6B).

Sensitivity of HSC to 4HNE toxicity and protein adduct formation

The initial viability level of HSC was determined to be 83%, which was maintained throughout 10 days of culture. Culture of HSC in the presence of G-CSF, SCF and IL-3 for 10 days resulted in a 20-fold increase in the number of viable CD34+ cells as measured by trypan blue exclusion (data not shown). Exposure of cultured HSC to 5 and 50 μM 4HNE resulted in 24 and 45% decreases, respectively, in cell viability after 1 h (Fig. 7A). An additional 1 h incubation of HSC in the presence of 4HNE did not significantly enhance 4HNE toxicity. No significant cytotoxic effects could be seen following addition of ethanol (vehicle) to the cultured cells. As observed in Fig. 7B, one or more major 4HNE-protein adducts in the molecular weight range of 60–70 kDa were observed in 4HNE-exposed cells. Quantitation of the 70-kDa band indicated a 2- and 10-fold increase in the intensity of this band after a 2-h incubation with 5 and 50 μM 4HNE, respectively.

Discussion

Although some excellent rodent models exist for developmental toxicity studies, the use of human cells and tissues were selected in the present study in order to draw meaningful conclusions regarding the links among the expression of human 4HNE-metabolizing enzymes and prenatal cell injury. In this regard, subtle differences in protein structure among relatively similar human and rodent biotransforma-

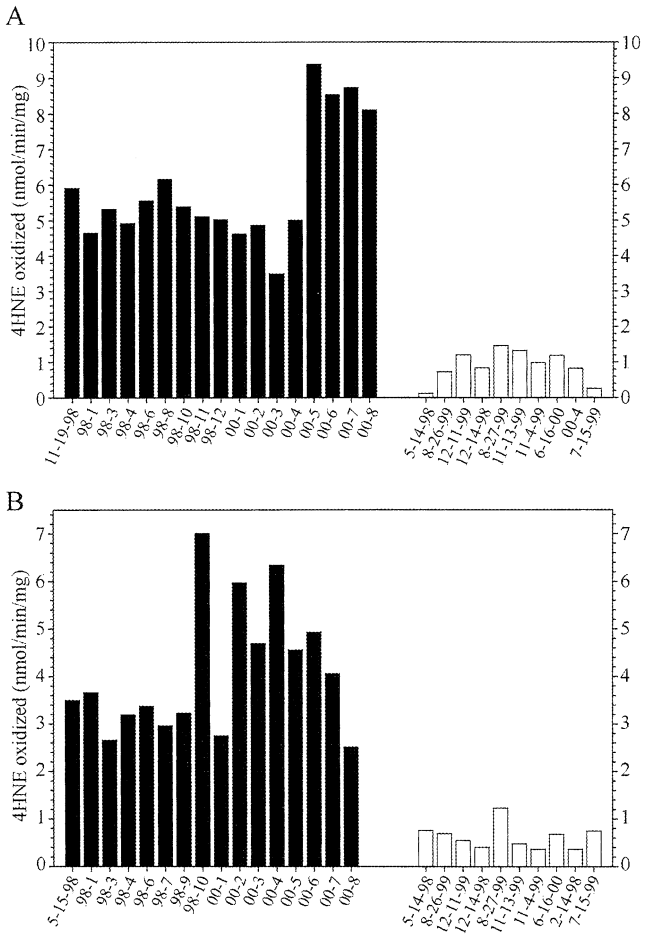

Fig. 4. 4HNE oxidation by ALDH activities in cytosolic and mitochondrial fractions. (A) Black bars indicate cytosolic ALDH-4HNE activities in a panel of adult liver cytosolic samples, and white bars indicate the same for prenatal liver cytosolic samples. (B) Mitochondrial ALDH-4HNE activities in adult liver mitochondrial samples (black bars) and prenatal liver mitochondrial samples (white bars). All assays were carried out in triplicate and corrected for nonenzymatic activity as described under Materials and Methods. The mean cytosolic and mitochondrial ALDH-4HNE activities were significantly lower among prenatal donors as compared to adults ($P \leq 0.05$).

tion proteins can result in marked differences in substrate recognition and catalytic efficiency. Furthermore, individual and developmental differences in protein expression in humans relative to rodents can be a further contributor to the uncertainty in species extrapolations. Our experiments using human-derived cell systems clearly demonstrate that during the second trimester, the human prenatal liver is relatively inefficient at detoxifying 4HNE via the predominant 4HNE metabolizing biochemical pathways at a substrate concentration of 100 μM 4HNE. The 4HNE concentration used in our in vitro assays was selected to allow for adequate product formation so that the various competing 4HNE metabolizing pathways could be detected and compared. However, lower and more physiologically relevant doses of 4HNE that fall below the K_ms of the 4HNE metabolizing enzyme may potentially yield somewhat different results than presented herein.

The rates of removal of 4HNE was generally low in both age groups, although a relatively high detection limit of the GST-4HNE assay relative to those for the 4HNE oxidative and reductive enzymes using nonpurified subcellular fractions was observed. In the case of GSTs, the rates of GSH-mediated conjugation of 4HNE would be influenced by the isoforms of GST expressed in prenatal liver. The relatively equivalent GST-4HNE activities observed in affinity-purified prenatal and adult liver fractions is consistent with our earlier study using a smaller sample size (Gallagher and Gardner, 2002) and the fact that two important human 4HNE-conjugating GST isoforms (e.g., hGSTA1 and hGSTA4) are expressed in both adult and second-trimester prenatal liver (Strange et al., 1989; Gallagher and Gardner, 2002). As observed, omission of GSH or heat inactivation of cyto-

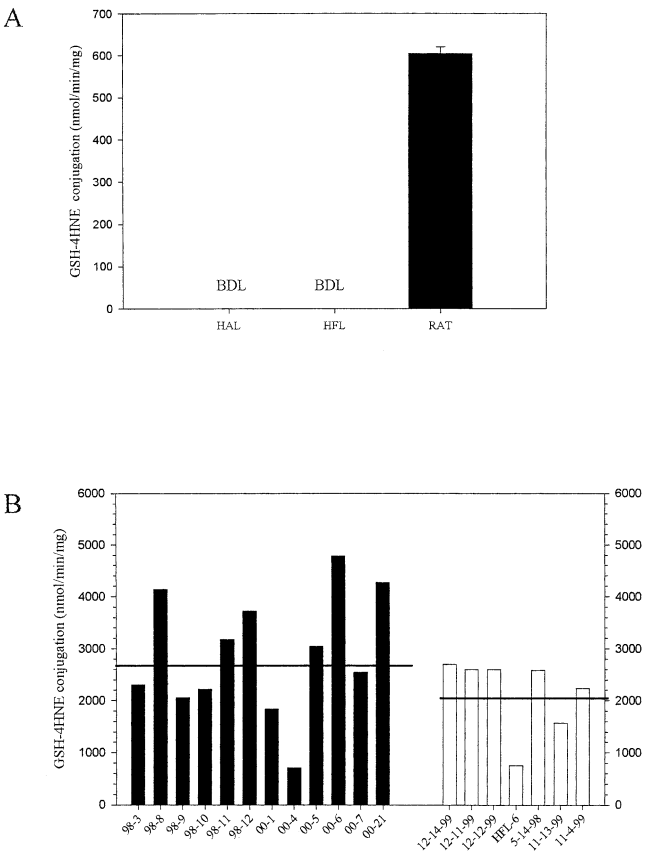

Fig. 5. Initial rates of GST-4HNE conjugation in (A) cytosolic fractions and (B) GSH affinity-purified cytosolic fractions from a panel of adult donors (black bars) and prenatal liver donors (white bars). Sprague–Dawley rat cytosol was used as a positive control for GST-4HNE activity in A. In A, GST-4HNE activity was below the practical limit of detection (10 nmol/min/mg) in adult liver cytosol (HAL) and human fetal liver cytosol (HFL). All assays were carried out in triplicate and corrected for nonenzymatic activity as described under Materials and Methods. The mean GST-4HNE activities in GSH-affinity purified fractions (as indicated by the horizontal bars) in B did not differ among the two age groups ($P \leq 0.05$).

Table 1
ALDH, ADH, ALRD, and GST activities toward 4HNE by prenatal liver hematopoietic stem cells

Subcellular fraction	ALRD	ADH	ALDH	GST
10,000*g* supernatant (S-9 fraction)	BDL*	BDL*	0.9	BDL**
10,000*g* pellet	NM	NM	4.4	NM

Note. Data represent the average of triplicate incubations (in nanomoles per minute per milligram). NM, not measured.

* BDL below practical limit of detection of ALRD and ADH activities (0.1 nmol/min/mg).

** BDL below practical limit of detection of GST-4HNE activity (10 nmol/min/mg).

solic fractions in the presence of GSH resulted in a dramatic loss of protection against 4HNE-protein adduct formation in fetal liver. Accordingly, among the competing biochemical pathways for 4HNE removal, it is likely that GSTs constitute the primary protective pathway against 4HNE injury in second trimester prenatal liver. Our observation of 5-fold lower initial rate ADH-4HNE activities in prenatal liver as compared to adults is consistent with reports showing developmentally controlled expression of ADH (Smith et al., 1971; Smith et al., 1972). When these early studies were performed, however, ADH2 (class II, suggested nomenclature of Duester et al., 1999) had not yet been discovered. With regard to ALDH2, our findings of prenatal liver mitochondrial ALDH-4HNE activity is consistent with the demonstrated expression of ALDH2 mRNA in a prenatal liver sample reported by Stewart et al. (1996). With regard to other potential pathways for 4HNE detoxification, rat liver NAD(P)H-dependent alkenal oxidoreductase may have an important role in reduction of 4HNE (Dick et al., 2001), but the human orthologue has not yet been characterized with regard to 4HNE biotransformation. Little if any previously reported ontogenic characterization has been carried out for ALRD activity. Further studies may help to better clarify the protective capacity of this enzyme in human prenatal tissues.

In addition to the important developmental differences in 4HNE detoxification pathways, we observed extensive interindividual differences in 4HNE biotransformation in both age groups. Although these observations are somewhat preliminary and could benefit from larger sample sizes, the variability observed in these rates may be reflected in a broad spectrum of susceptibility to reactive alkenals such as 4HNE. Consistent with this hypothesis, McCarver et al. demonstrated that the ADH2*3 polymorphism, found in high frequency among certain ethnicities, increased the risk of teratogenesis as a result of maternal ethanol exposure (McCarver, 2001). Similarly, Sjörgen–Larsson syndrome has been attributed to mutations in the *ALDH3A2* gene, the protein product of which contributes to ALDH oxidation of fatty and aromatic aldehydes (Rizzo et al., 1999). Interestingly, a search of the National Center for Biotechnology Single Nucleotide Polymorphism (SNP) database revealed the presence of approximately 400 SNPs associated with human ADH genes. Although most of these SNPs will likely not result in mutant proteins with altered kinetic characteristics, it is likely that new allelic variants that influence susceptibility to oxidative injury in ADH, as well as ALRD, ALDH, and GST, will be identified.

As observed in the present study, the relatively slow initial rates of 4HNE conjugation in subcellular fractions of prenatal liver likely contributed to the sensitivity of 4HNE injury seen using two different in vitro systems and cell models. Our experiment demonstrating that human prenatal liver cytosol was less efficient at attenuating 4HNE–protein adduct formation in vitro served as an overall index of protein alkylation, which is strongly linked to the development of hepatotoxicity (Gregus and Klaasen, 1996; Wells et al., 1997). This linkage is further strengthened by our observation that at a physiologically relevant dose of 5 μM, 4HNE produced a 24% loss of viability as measured by Alamar blue reduction to cultures of human HSCs after 1 h of exposure. The Alamar blue assay is a sensitive indicator of the reducing environment of proliferating cells and is highly correlated to results of tetrazolium salts such as MTT and XTT (Takahashi et al., 1997). However, Alamar blue offers the advantage in that it is highly stable under cell culture conditions and is nontoxic, so that continuous monitoring of cells in culture is permitted. The fact that less than a nonlinear ($<$twofold) increase in toxicity was observed in HSC cultured in the presence of 50 μM 4HNE, as opposed to 5 μM 4HNE, suggests that the predominant 4HNE metabolizing enzymes in HSC may have an overall high K_m, leading to rapid clearance of 4HNE at the relatively higher 4HNE dose. The fact that 5 μM 4HNE decreased the viability of HSC places these cells among the more sensitive to 4HNE toxicity (Esterbauer et al., 1991). Although little is known regarding the expression of 4HNE detoxification pathways in human fetal liver HSCs, mRNAs encoding two important 4HNE-metabolizing GST isozymes (hGSTA1 and hGSTA4) have been shown to be present in human hematopoietic cell lines (Wang et al., 2000). However, it is possible that the level of the active 4HNE-metabolizing GST proteins may be present at relatively low levels in these cells. If so, the rate of conjugation of endogenously generated 4HNE in these cell targets may not be sufficient to protect against injury.

The findings presented here suggest that prenatal liver HSC may be a target of 4HNE and possibly other reactive aldehydes produced by transplacental oxidants. In this regard, the generally low level of ADH-4HNE and GST-4HNE activities in HSC likely contributed to the high sensitivity of HSCs to 4HNE-mediated protein alkylation and loss of viability. The appearance of one or more protein targets of approximately 70 kDa is suggestive of specific subcellular targets of covalent modification by 4HNE within

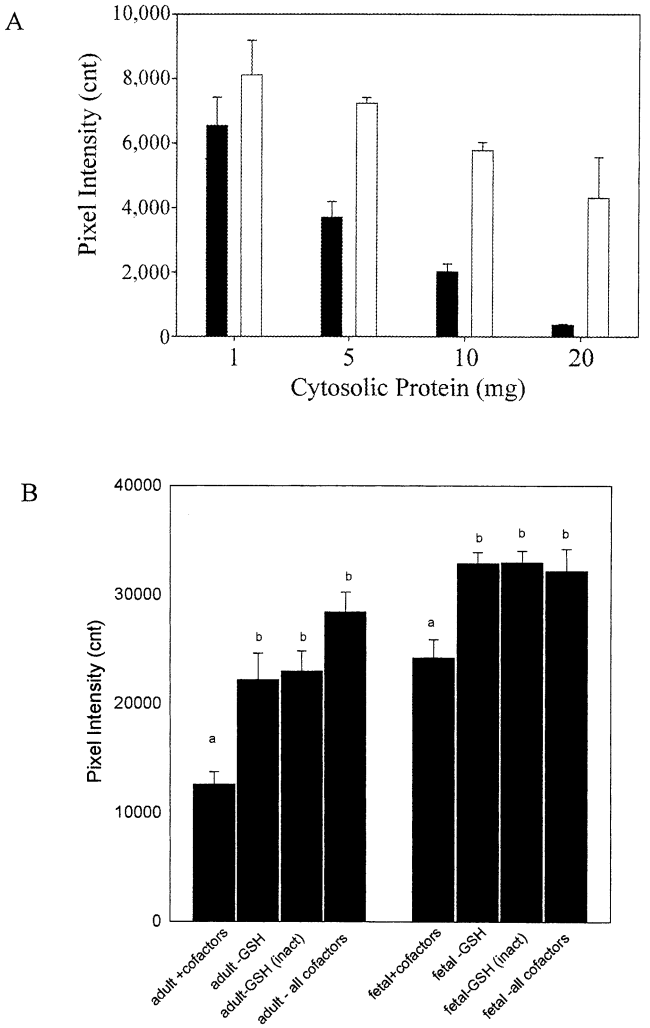

Fig. 6. (A) Comparative inhibition of 4HNE–protein adduct formation by adult and prenatal liver cytosol. Black bars indicate average intensities of 4HNE–protein adduct antibody reactivity at various levels of adult liver cytosol. White bars indicate the same reactions using prenatal liver cytosol. Error bars indicate standard error of three or more replicates. Results presented are from a typical experiment that was representative of repeated experiments. (B) Effect of omission of GSH on the comparative inhibition of 4HNE–protein adduct formation by adult and prenatal liver cytosol. Reactions were conducted as described in A with 2.5 μg of adult or prenatal liver cytosol in the presence of all cofactors except GSH. Reactions were also carried out in the presence of GSH and using heat-inactivated cytosols. Groups sharing different letter asterisks are significantly different at $P \leq 0.05$.

human prenatal liver HSCs. These targets are currently being identified for their possible roles in the high degree of sensitivity that was observed in these stem cells. Relevant to this concept are previous findings that 4HNE appears to mediate apoptosis as a function of ethanol exposure in the brain of the prenatal rat (Ramachandran et al., 2001). In rats, this apoptotic event coincides with adduction of cytochrome *c* oxidase (COX) by 4HNE and malondialdehyde following ethanol intake (Chen et al., 1999; Chen et al., 2000). However, the size of the primary adduct seen in our experiments would seem to preclude a subunit of COX to be the actual target (Chen et al., 1999). The formation of 4 HNE–protein adducts has been reported in several biological systems, including oxidized hepatocytes, renal proximal tubules of rats treated nitrilotriacetate, and in lung lavage cells following in vivo ozone exposure. Hartley et al. (1997) demonstrated that aldehyde-modified proteins

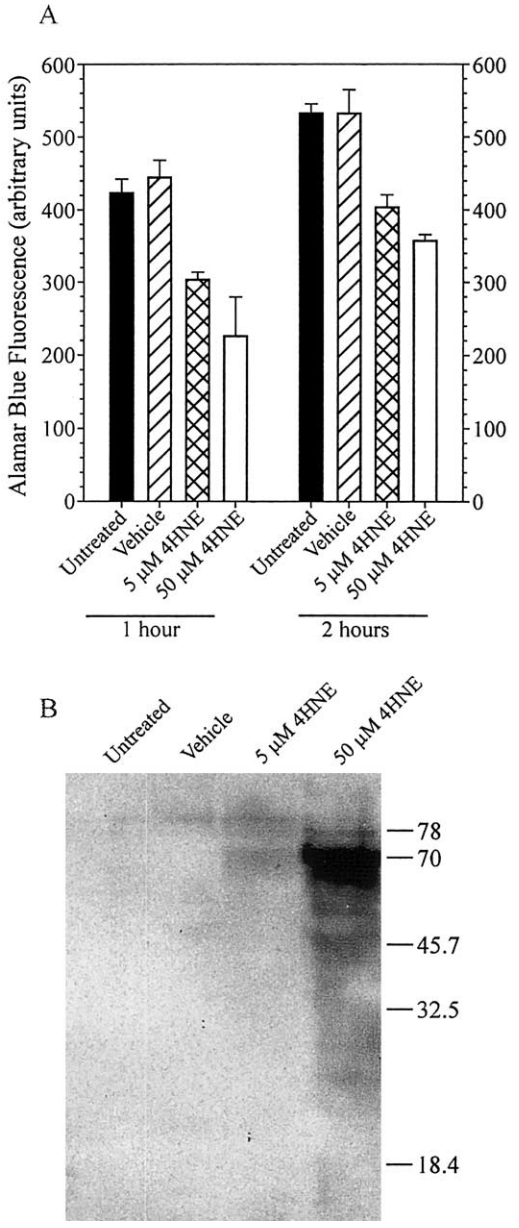

Fig. 7. Sensitivity of cultured HSC to 4HNE toxicity and 4HNE–protein adduct formation. (A) Viability falls sharply upon treatment with low concentrations (5 μM) of 4HNE. Error bars indicate standard error of three experiments. (B) SDS–PAGE followed by western analysis using a 4HNE-protein adduct antibody reveals formation of high molecular weight 4HNE-protein adducts with discrete banding. Electrophoretic standards were used to discern the noted molecular weights (kDa); molecular weight of the primary band was subsequently interpolated.

produced in hepatocytes in the presence of carbon tetrachloride may not clearly underlie cell death or be an immediate consequence of the protein alkylation by malondialdehyde or 4HNE. We are currently identifying the covalent targets of 4HNE and also determining if the 4HNE-covalent adducts elicited any adverse consequences to HSC differentiation.

The critical hematopoietic function of HSC and the ramifications of 4HNE injury to these targets may be particularly relevant to the potential etiology or progression of blood-borne malignancies. In this regard, mutational events in HSC may have particular relevance to the risk of developing certain childhood cancers such as acute lymphoblastic leukemia (ALL). Other investigators have noted that its prevalence among children probably results in part from the fact that normal HSCs share certain common characteristics with malignant cells, including ability to proliferate, survive vascular transit, transmigrate into tissues, and grow in semisolid media (Irons and Stillman, 1996). Although childhood leukemias such as ALL have been widely studied, a clear epidemiological link between the disease and human genetics has only begun to be established. In this regard, Krajinovic et al. (1999) showed that specific genotypes of GSTs and cytochromes P450 were significant predictors of ALL risk. Furthermore, maternal occupational exposures to pesticides and certain industrial compounds were linked to increased risk of developing leukemia in children (Shu et al., 1996, 1999). The presence of many confounding factors and the high degree of interindividual variability of xenobiotic metabolism, however, may account for some of the difficulty that researchers have had in establishing a clear causal relationship among biotransformation enzyme expression, chemical exposure, and risk for developing certain leukemias.

In summary, during the second trimester, human prenatal liver cells are highly sensitive to in vitro oxidative injury arising from 4HNE production. This sensitivity appears to be largely due to a poor ability to remove 4HNE by aldehyde metabolizing protective enzymatic pathways during this stage of development. Although our in vitro studies suggest that there is less protection afforded to prenatal tissues during oxidative injury, the relevance of these studies to the in vivo condition has not been established. Our findings using cultured HSCs offer potentially important information with regard to the development of perinatal blood-borne diseases that might have in utero injury as a causative factor. In this regard, it will be important to characterize the potential for alterations and HSC differentiation and gene expression in the presence of physiologically relevant levels of reactive metabolites, as well as to identify protein targets of aldehyde injury in these functionally important progenitor cells.

Acknowledgments

This work was supported in part by grants from NIH (R01-ES09427) and the U.S. Environmental Protection Agency (STAR R8274401).

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